PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Eva KONTSEKOVA	Group Art Unit: 1633
Serial No.: 10/521,140	Examiner: Chernyshev, Olga N.
Filed: October 31, 2005	Atty. Dkt. No.: SONN:065US
For: TRUNCATED TAU PROTEINS	

DECLARATION OF MICHAL NOVÁK, M.D.V., Ph.D., D.Sc.

I, Michal Novák, hereby declare as follows:

1. I am a citizen of the Slovak Republic residing at Novosvetska 27, 81104 Bratislava, Slovak Republic.

2. I am currently the Chief Scientific Officer at Axon Neuroscience, GmbH, which is the assignee of the above-referenced patent application. I am also a co-author of Novak *et al.* (EMBO J., 12:367-370 (1993)) and Vechterova *et al.* (Neuroreport, 14(1):87-91 (2003)). A copy of my *Curriculum Vitae* is attached as Exhibit 1. The Novak and Vechterova publications are attached as Exhibits 2 and 3, respectively.

3. I am providing this declaration to provide evidence that the tau core fragment, which is also referred to as dGAE, disclosed in WO 96/30766, Novak *et al.* (1993), and Vechterova *et al.* (2003) is conformationally different from the tau type IA molecule (SEQ ID NO: 1) disclosed in the above-referenced patent application.

4. This Declaration contains the results of studies comparing the ability of monoclonal antibodies DC-11, DC-25, and DC-44, to bind to normal tau and truncated tau. The DC-11 monoclonal antibody specifically binds to a truncated tau molecule, but does not bind to normal human tau protein (Vechterova, Abstract). DC-44 is monoclonal antibody that also specifically binds to a truncated tau molecule, but does not bind to normal human tau protein. DC-25 is a pan-tau antibody that binds to both normal and truncated tau molecules. The studies described in this Declaration were performed under my direction in my laboratory.

5. As shown in the figures in Exhibit 4, tau type IA is conformationally different from the tau core fragment disclosed in WO 96/30766. Figure 1 shows immunoblotting with mAb DC-25 (left panel), which recognizes all tau forms, and conformation-specific mAb DC-11 (Acc. No. 00082216) (right panel). Tau protein (80 ng/lane) was separated on a 5-20% SDS-PAGE and transferred onto PVDF membrane and subsequently detected with the respective monoclonal antibodies according to standard Western blot methods. Lane 1 in both the DC-25 and DC-11 panels shows brain derived tau type IA. Lane 2 in both the DC-25 and DC-11 panels shows recombinant dGAE. As shown in Figure 1, the DC25 antibody detected both brain derived tau type IA and recombinant dGAE. In contrast, conformation-specific mAb DC-11 detected brain derived tau type IA, but did not detect recombinant dGAE even though these molecules have the same primary structure.

6. Figure 2 shows discrimination between tau type IA (SEQ ID NO: 1) and dGAE by monoclonal antibodies DC44 (Acc. No. 02060767) and DC11 (Acc. No. 00082216) by ELISA even though these two molecules have identical primary structure. Native (tau type IA, SEQ ID NO: 1) and recombinant (dGAE) forms of tau protein were at the same concentration (5ug/ml). Monoclonal antibodies DC-44 and DC-11 discriminated between recombinant dGAE and tau

type IA fragments derived from brain tissue. Only the native forms of tau isolated from Alzheimer's diseased brain tissue were recognized whereas dGAE yielded only background signals in this assay. As a control monoclonal antibody DC-25 was used, which recognizes both forms of the tau protein.

7. In conclusion, the data presented in this Declaration demonstrates that the tau core fragment disclosed in WO 96/30766 is conformationally different from the tau type IA molecule (SEQ ID NO: 1) disclosed in the above-referenced patent application.

8.I declare that all statements made of my knowledge are true and all statements made on the information are believed to be true; and, further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereupon.

Date: 06.06.2008

Michal Novák, M.D.V., Ph.D., D.Sc.

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EXHIBIT 1

CURRICULUM VITAE

Name:
Prof. Novák Michal, MDV, PhD, DSc

Official address:
Institute of Neuroimmunology, Slovak Academy of Sciences, 842 46 Bratislava, Slovak Republic.

Date and place of birth:
September 29, 1947 - Bratislava (Slovak Rep.)

Nationality:
Slovak

Education (degrees, dates, universities)

M.D.V.	doctor of vet.medicine 1971, First class honours, University of Veterinary Medicine, Kosice, Slovak Republik
Ph.D. - 1978	Univ.Vet.Medicine, Kosice, Slovak Republic, (molecular immunology)
1978-1980	Post doctoral fellow, Slovak Academy of Sciences, Bratislava,Slovak Republik
1980-1985	Group leader, Slovak Academy of Sciences, Bratislava, Slovak Republik
1985-1987	Senior Research Scientist - (24 months)MRC Laboratory of Molecular Biology, Cambridge, England (working with A.Klug/C.Milstein)
1988 (6 months)	MRC Laboratory of Molecular Biology, Cambridge, England (working with sir A.Klug and C.Milstein)
1989 (6months)	University of Cambridge, Dept. Psychiatry, Cambridge, England (working with sir M.Roth)
1990-1994	MRC Laboratory of Molecular Biology, Cambridge, England (working with sir A.Klug /C.Milstein /sir M.Roth/J.Walker)
D.Sc - 1994	Neurosciences - Slovak Academy of Sciences, Bratislava.

Career/Employment (employers, positions and dates)

1978-1980	Part time Assistant Professor, Dept. Microbiol.Immunology, Univ.Vet.Medicine, Kosice, Slovakia
1980-1985	Associate Professor of Molecular Neurobiol.Immunology, Slovak Academy of Sciences, Bratislava
1985-1994	Senior Research Scientist, MRC, Laboratory of Molecular Biology and Univesity of Cambridge, Cambridge, England
1994- 2000	Visiting Professor of Neurosciences, International School for Advanced Studies (ISAS), Trieste,Italy International Scholar of Howard Hughes Medical Institute, Maryland, USA
1994-	Foundation Memory, Chairman (Founder)
1995-2000	Full Professor of Microbiology and Immunology, University of Veteterinary Medicine, Kosice, Slovak Republic
1996	Director (Founder) , Institute of Neuroimmunology, Slovak Academy of Sciences
1997-	Member - Council of Goverment for Science
1998-	President (founder) Slovak Alzheimer's Society, member of Alzheimer's Disease International (ADI) and Alzheimer Europe (AE)
1999-	Axon Neurosciences,GmbH, Chief Scientific Officer, Vienna Austria

Specialization

(i) main field
Molecular Neurobiology
Neurosciences
(ii) other field
Molecular Immunology
(iii) current research interest
Role of tau proteins in Alzheimer's disease pathogenesis
Diagnostic assays for AD and animal models
Engineering of monoclonal antibodies as structural biomedical probes

Honours, Awards, Fellowships, Membership of Professional Societies

1985 - National Prize for Medicine, Bratislava, Slovakia
1994 - 1996 Grant from Human Frontiers Science Organisation, (HFSPO) Strasbourg.
1995-2000 Grant from Howard Hughes Medical Institute, Maryland,USA

Membership: Society for Neuroscience, Society for Immunology, New York Academy of Sciences.

Member of Editorial Boards:

Alzheimer's Reports (CC listed)
Brain Aging
Bratislava Medical Journal

(Citations in CC – around 2000)
Selected List of Research Publications
Michal Novák

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EXHIBIT 2

The EMBO Journal vol.12 no.1 pp.365–370, 1993

Molecular characterization of the minimal protease resistant tau unit of the Alzheimer's disease paired helical filament

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Communicated by A.Klug

The Alzheimer's disease paired helical filament (PHF), after digestion with Pronase, retains its characteristic morphological features. We term this the protease resistant core PHF. A 12 kDa tau fragment can be released from the core as an essentially pure preparation. Sequence analysis of this fragment revealed six distinct N-termini beginning in the repeat region of tau. The precise C-terminus is unknown, but the fragment is ~100 residues long. A monoclonal antibody, mAb 423, which recognizes the core PHF and the 12 kDa tau fragment, does not recognize normal full-length tau. We describe cDNA synthesis and expression of candidate 12 kDa tau analogues which permit the mapping of the mAb 423 epitope. mAb 423 recognizes all and only those analogues which terminate at Glu391, which lies beyond the homology repeat region. Addition or removal of a single residue at the C-terminus abolishes immunoreactivity. Therefore, mAb 423, together with knowledge of the N-terminus, can be used to measure the precise extent of 12 kDa PHF core tau fragment which we term the minimal protease resistant tau unit of the core PHF. This unit is 93–95 residues long, which is equivalent to three repeats, but is 14–16 residues out of phase with respect to the maximum homology organization of the repeat region. mAb 423 labels isolated PHFs prior to Pronase digestion and intracellular granular and neurofibrillary degeneration in Alzheimer's disease tissues. The constraints which determine endogenous truncation at Glu391 appear to be characteristic of an assembled configuration of tau, either within the PHF or its precursor.

Key words: Alzheimer's disease/paired helical filament/protease resistant tau unit/tau protein

Introduction

Tau protein normally functions as an axon-specific, microtubule associated protein. The capacity to form reversible interactions with tubulin is required for the normal physiology of the neuronal cytoskeleton. In Alzheimer's disease, tau protein accumulates abnormally in the somatodendritic compartment, particularly of cortical pyramidal cells. These accumulations take the form of neurofibrillary tangles which contain abnormal fibres called paired helical filaments (PHFs). Proof that tau is an integral part of the PHF came from our earlier work on the protease resistant PHF, which we termed the 'PHF core'. This structure was chosen because it retains all the characteristic morphological features of the PHF, apart from the loss of a protease-sensitive outer coat (Wischik *et al.*, 1988a,b). We reported a method for releasing an essentially pure preparation of a 12 kDa tau fragment from the core PHF preparation. The link between this fragment and the core PHF was established by immunoelectron microscopy and immunoblotting using a monoclonal antibody (mAb 423) which is selective for PHF-tau (Wischik *et al.*, 1988a; Novak *et al.*, 1989).

Detailed sequence analysis of the 12 kDa species showed that it consists of six N-terminally distinct tau peptides derived from the repeat region of 3- and 4-repeat tau isoforms, but restricted to approximately three repeats regardless of isoform (Jakes *et al.*, 1991). This fragment is recognized by mAb 423 as immunochemically different from normal isolated or expressed human tau. We have previously shown that this immunospecificity is independent of phosphorylation (Novak *et al.*, 1991).

In this study we report production of a set of tau fragments from mRNA isolated from Alzheimer's disease brain tissues by reverse transcription and cDNA PCR amplification. Sense PCR primers were designed so as to reproduce the known N-terminal heterogeneity of species present in the 12 kDa band. In view of earlier evidence that the C-terminus of the 12 kDa fragment is critical for mAb 423 immunoreactivity (Novak *et al.*, 1991), antisense primers were chosen to test various candidate C-terminal truncation points which might be responsible for immunoreactivity. We now report that mAb 423 recognizes a specific C-terminal cleavage site at Glu391. These data, together with previously established positions of the N-termini, serve to define the extent of the minimal protease-resistant tau unit of the core PHF. This unit is 93–95 residues long, the equivalent of three repeats, but is 14–16 residues out of phase with respect to the maximum homology organization of the repeat region. mAb 423 immunoreactivity can be demonstrated in isolated PHFs prior to Pronase digestion, and in intracellular granular and neurofibrillary degeneration in Alzheimer's disease tissues not treated with exogenous proteases. We propose that truncation of tau at Glu391 is produced by endogenous proteases as one of the events leading to PHF assembly, or alternatively that it reflects the action of proteases on a partially assembled precursor.

Results

Production of candidate PHF core tau analogues

Our previous studies showed that the 12 kDa fragment released from the protease-resistant core of the PHF consists of six N-terminally distinct peptides derived from the repeat region of 3- and 4-repeat tau isoforms, but encompassing approximately three repeats (Jakes *et al.*, 1991). These are

denoted by the letters a–d in Figure 1. In the present study we designed tau fragments with N-termini corresponding to species denoted by a–d, representing repeats R1, R3 and R4 from 3-repeat tau (a and b) and repeats R2, R3 and R4 from 4-repeat tau (c and d). In view of earlier data showing that the C-terminus is critical for mAb 423 immunoreactivity (Novak *et al.*, 1991), species a and d were designed so as to test the possible contribution from the five different C-termini shown in Figure 1. In the case of the a species, these are denoted by a/KAKT, a/GA, a/GAE, a/GAEI and a/KSPV. The corresponding d species are denoted by d/KAKT, d/GA, d/GAE, d/GAEI and d/KSPV. Species b and c were expressed only as b/GAE and c/GAE.

cDNA derived from mRNA extracted from Alzheimer's disease brain tissue was used as template for PCR. Primers were designed such that primer A (sense) contained an *Nde*I

I.

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                266           280        290        300        310        320        330
       ┌ a/KAKT --HQPGGGK-------------------------------VQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQV
       │ a/GA   --HQPGGGK-------------------------------VQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQV
3rpt  ┤ a/GAE  --HQPGGGK-------------------------------VQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQV
       │ a/GAEI --HQPGGGK-------------------------------VQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQV
       │ a/KSPV --HQPGGGK-------------------------------VQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQV
       └ b/GAE  LKHQPGGGK-------------------------------VQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQV
       ┌ c/GAE  ---------------------------------HVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQV
       │ d/KAKT -------------------------------IKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQV
4rpt  ┤ d/GA   -------------------------------IKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQV
       │ d/GAE  -------------------------------IKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQV
       │ d/GAEI -------------------------------IKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQV
       └ d/KSPV -------------------------------IKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQV
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                340       350       360       370       380       390                AA     MW
       ┌ a/KAKT EVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKT                  (88)    9479
       │ a/GA   EVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGA              (92)    9859
3rpt  ┤ a/GAE  EVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAE             (93)    9988
       │ a/GAEI EVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAEI            (94)   10101
       │ a/KSPV EVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYKSPV     (100)   10774
       └ b/GAE  EVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAE             (95)   10229
       ┌ c/GAE  EVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAE             (93)    9918
       │ d/KAKT EVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKT                  (90)    9650
4rpt  ┤ d/GA   EVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGA              (94)   10030
       │ d/GAE  EVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAE             (95)   10159
       │ d/GAEI EVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAEI            (96)   10272
       └ d/KSPV EVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYKSPV     (102)   10945
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II.

A [R1,R3,R4]

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                                      D L K N V K S K I G S T E N
↓L K↓H Q P G G G K V Q I V Y K P V D L S K V T S K C G S L G N
 I H H K P G G G Q V E V K S E K L D F KDR V Q S K I G S L D N
 I T H V P G G G N K K I E T H K L t f ren a k a k t d h g a e↓
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B [R1,R2,R3]

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                                      D L K N V K S K I G S T E N
↓L K↓H Q P G G G K V Q I I N K K L D L S N V Q S K C G S K D N
 I K H V P G G G S V Q I V Y K P V D L S K V T S K C G S L G N
 I H H K P G G G Q V E V K S E K L D F KDR V Q S K I G S L D N?
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C [R2,R3,R4]

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                                      D L S N V Q S K C G S K D N
↓I K↓H V P G G G S V Q I V Y K P V D L S K V T S K C G S L G N
 I H H K P G G G Q V E V K S E K L D F KDR V Q S K I G S L D N
 I T H V P G G G N K K I E T H K L t f ren a k a k t d h g a e↓
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Fig. 1. I. N-terminal sequence analysis of the 12 kDa unit released from protease-resistant PHF core preparations has shown the presence of six distinct peptides derived from the repeat region of 3- and 4-repeat tau isoforms. Four of these are designated a–d (Jakes *et al.*, 1991) and are aligned according to the known cDNA sequence of the longest human tau isoform (Goedert *et al.*, 1989a). Species a and d were designed so as to test the role of the five different C-termini shown in determining mAb 423 immunoreactivity. Species b and c were expressed with a single C-terminus. The residue lengths and predicted molecular weights are also shown. **II.** The six N-termini of the native 12 kDa tau unit (shown by arrows). These can be grouped into three pairs, derived from 3-repeat (A: R1-R3-R4) or 4-repeat (B: R1-R2-R3 or C: R2-R3-R4) isoforms. mAb 423 immunoreactivity serves to define a C-terminal boundary at Glu391 (shown by arrow). The N- and C-terminal boundaries thus serve to define a phasing of the tandem repeat region within the PHF core which is shifted 14–16 residues with respect to the sequence homology repeats (shown in upper case letters) defined by Goedert *et al.* (1989b). The unit is 93–95 residues long which is equivalent to three tandem repeats. We predict according to this pattern that the species B (R1-R2-R3) terminates at a homologous position. The boundaries of the unit are also out of phase with the tubulin binding domains proposed by Butner and Kirschner (1991), which are shown underlined.

site and primer B (antisense) an *Eco*RI site. This made it possible to subclone PCR fragments downstream of the T7 RNA polymerase promoter in the expression vector pRK172 (McLeod *et al.*, 1987). The authenticity of all DNA fragments used for expression was confirmed by full length sequencing of both strands.

Expression products were purified to >95% purity by ion exchange chromatography (Goedert and Jakes, 1990). The authenticity of the expressed purified products was checked by N-terminal amino acid sequencing, and confirmed by immunoreactivity with the generic anti-tau antibody mAb 7.51 (Novak *et al.*, 1991).

The actual gel mobility observed on SDS–PAGE did not correspond to that predicted for fragments with lengths ranging from 88 to 102 residues. Species a/KSPV and d/KSPV had apparent gel mobilities of 14 kDa (Figure 2, lanes 11, 12). Species a/KAKT, d/KAKT, d/GA, a/GAE, b/GAE, c/GAE, d/GAE, a/GAEI, d/GAEI had gel mobilities closer to 12 kDa (Figure 2, lanes 2–10).

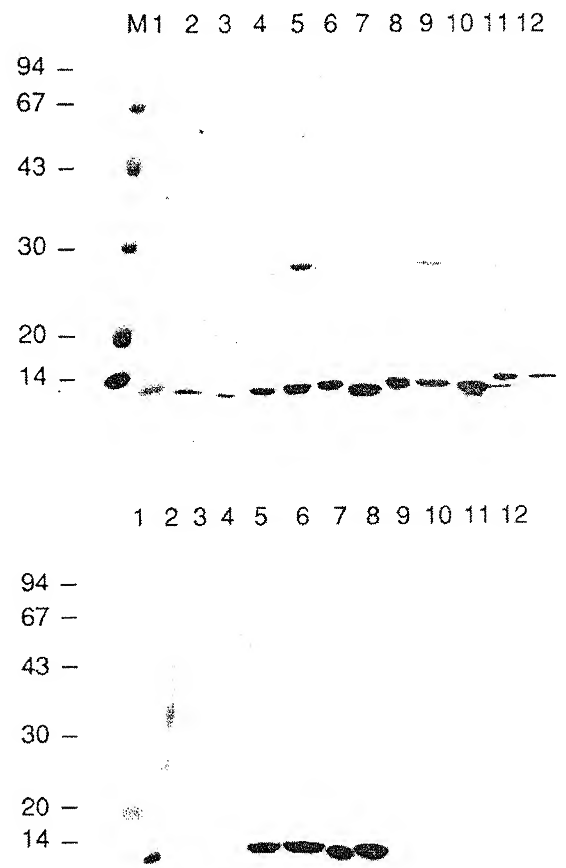

Fig. 2. Above: Coomassie stained gel of the expressed PHF core tau analogues used in this study. Lane 1 shows the authentic 12 kDa unit released from PHF core preparations for comparison. Lanes 2–12, containing the species denoted according to the nomenclature shown in Figure 1, are: 2, a/KAKT; 3, d/KAKT; 4, d/GA; 5, a/GAE; 6, b/GAE; 7, c/GAE; 8, d/GAE; 9, a/GAEI; 10, d/GAEI; 11, a/KSPV; and 12, d/KSPV. The expressed analogues are arranged in order of ascending molecular weight. The species a/GAE, b/GAE, c/GAE, d/GAE, a/GAEI and a/KSPV form multimers whose apparent mobilities are not necessarily integral multiples of 12 kDa. **Below**: mAb 423 recognizes all and only the species terminating in GAE irrespective of the N-terminus (lanes 5, a/GAE; 6, b/GAE; 7, c/GAE; and 8, d/GAE) and the native 12 kDa tau unit (lane 2). Removal of a single residue [d/GA (lane 3)] or addition of a single residue [a/GAEI (lane 9) or d/GAEI (lane 10)] is sufficient to abolish immunoreactivity. Further removal [a/KAKT (lane 3)] or addition [a/KSPV or d/KSPV (lanes 11 and 12 respectively)] of residues likewise produces negative fragments. The multimeric forms of species terminating at GAE are not recognized by mAb 423.

mAb 423 epitope

Immunoblotting of the candidate PHF core tau analogues we had expressed showed that four of the 12 fragments were recognized by mAb 423. These were a/GAE, b/GAE, c/GAE and d/GAE (Figure 2). As seen in Figure 1, these fragments differ at the N-terminus by two to four residues at the positions underlined: a, HQPGGGKV; b, LKHQPGGGKV; c, HVPGGGSV; and d, IKHVPGGGSV. However, the mAb 423 reactive fragments are identical at the C-terminus, ending at Glu391.

The remaining expressed tau fragments with identical N-termini, but different C-termini, were all negative. In particular the failure of mAb 423 to recognize a/GA, d/GA, a/GAEI, d/GAEI indicates that addition or removal of a single residue is sufficient to abolish mAb 423 immunoreactivity regardless of the N-terminus. It is therefore not surprising that fragments a/KAKT and d/KAKT, which are five residues shorter, and a/KSPV and d/KSPV, which are seven residues longer, were not recognized by mAb 423 (Figure 2). Thus mAb 423 recognizes all and only those fragments which terminate at Glu391.

Boundaries of the minimal protease-resistant tau unit of the core PHF define a characteristic phasing of the tandem repeat region of tau

It is now possible to define the region of tau that forms the minimal protease-resistant unit of the core PHF. The six N-termini, determined previously by direct sequence analysis (Jakes *et al.*, 1991), can be grouped into three pairs (Figure 1A–C), derived from 3-repeat (A: R1-R3-R4) or 4-repeat (B: R1-R2-R3 or C: R2-R3-R4) tau isoforms, but encompassing only 3-repeats. mAb 423 measures the C-terminal extent of the unit in the case of species A and C (Figure 1). Since all run as a single electrophoretic band at 12 kDa, we predict that species B terminates at or close to Asn351 according to the general pattern shown in Figure 1A and C. The minimal core tau unit is 93–95 residues long and is 14–16 residues out of phase with respect to the maximum homology alignment proposed by Goedert *et al.*, (1989b). The unit is also out of phase with the linker-binder domain organization proposed by Butner and Kirschner (1991).

Immunoelectron microscopy of PHFs isolated with and without Pronase digestion

mAb 423 immunoreactivity can be demonstrated in the form of granular deposits (Mena *et al.*, 1991) and granulovacuolar degeneration (Bondareff *et al.*, 1991), and in intracellular neurofibrillary tangles (Bondareff *et al.*, 1990) in Alzheimer's disease tissues not treated with proteases. In order to confirm that endogenous truncation of tau occurs in the course of neurofibrillary degeneration, we have re-examined the labelling by mAb 423 of PHFs isolated without or with Pronase digestion (Wischik *et al.*, 1988b). Figure 3 shows immunogold labelling of PHFs with mAb 423 at 10 μg/ml and below. Labelling of non-Pronase treated PHFs

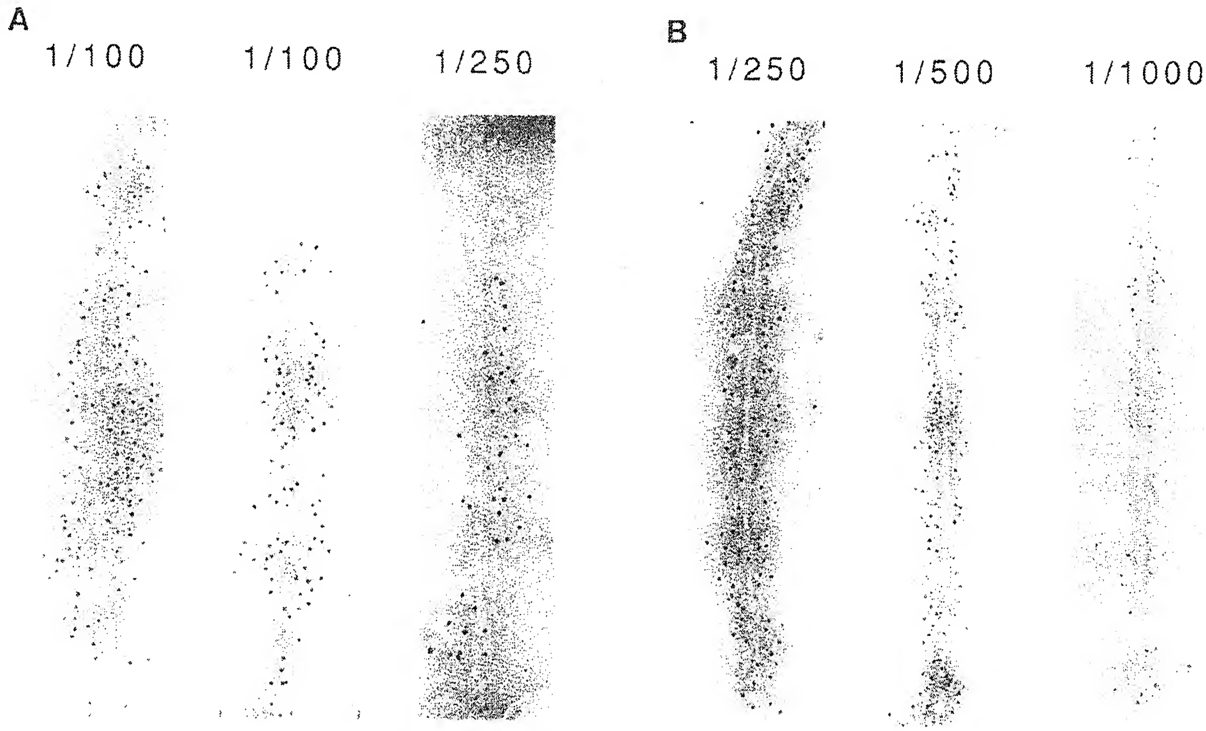

Fig. 3. Immunogold labelling by mAb 423 of PHFs isolated without (A) and with (B) Pronase digestion. Starting with a stock solution of mAb 423 (1 mg/ml), labelling of PHFs isolated without Pronase digestion was observed at dilutions of 1/100 (10 μg/ml) and 1/250 (4 μg/ml). Weak labelling of occasional PHFs was also observed at 1/500 (2 μg/ml, not shown). No labelling can be seen when the mAb 423 culture supernatant is used directly, implying that the antibody concentration is <2 μg/ml. By contrast, strong labelling of Pronase digested PHFs was observed at 1/250 (4 μg/ml), 1/500 (2 μg/ml) and 1/1000 (1 μg/ml) dilutions. Thus, Pronase digestion enhances labelling of PHFs, but the truncation at Glu391 is already present in non-Pronase PHFs.

by mAb 423 indicates that PHFs already contain tau truncated at Glu391 (Figure 3A). Pronase digestion, which is known to remove N-terminal tau epitopes (Brion *et al.*, 1991), substantially enhances mAb 423 labelling (Figure 3B). This enhancement may be due either to removal of occluding portions of the tau molecule or to further truncation of tau at Glu391 by exogenous protease. The constraints which determine truncation at Glu391 appear to be characteristic of an assembled configuration of tau, within either the PHF or its precursor.

Discussion

The mechanisms by which tau protein is modified to form highly regular assemblies in Alzheimer's disease is unknown. In our earlier work (Novak *et al.*, 1989) we described a monoclonal antibody (mAb 423) which labels the PHF core in immunoelectron microscopy and recognizes in immunoblots an essentially pure preparation of a 12 kDa tau fragment released from bulk protease-resistant core PHF preparations from Alzheimer's disease brain tissues (Wischik *et al.*, 1988a). mAb 423 distinguishes between this fragment and normal isolated or expressed human tau (Novak *et al.*, 1991). The selectivity of mAb 423 has been useful in several histological (Bondareff *et al.*, 1990, 1991; Mena *et al.*, 1991), ultrastructural (Wischik *et al.*, 1998b; Novak *et al.*, 1991) and biochemical (Harrington *et al.*, 1991; Mukaetova-Ladinska *et al.*, 1992) studies of neurofibrillary pathology in Alzheimer's disease.

Our earlier work on the determinants of mAb 423 immunoreactivity showed that it was not affected by dephosphorylation, and suggested that both N- and C-terminal parts of the 12 kDa fragment were required. In the present study, we expressed a range of candidate PHF core tau analogues to determine whether the truncation of the core tau unit in the 12 kDa fragment could account entirely for the immunochemical difference between normal tau and PHF-tau detected by mAb 423. The essential result of this study is that mAb 423 recognizes a specific C-terminal cleavage of tau at Glu391, and that the conformation of the fragment does not contribute to immunoreactivity as previously thought (Novak *et al.*, 1991). mAb 423 recognizes all and only those fragments which terminate at Glu391, and removal or addition of a single residue is sufficient to abolish immunoreactivity. These results effectively rule out the possibility that any modification other than specific C-terminal truncation is required for recognition by mAb 423.

The body of results now available serves to define the precise boundaries of the minimal protease resistant tau unit of the core PHF. The N-termini have been established directly by sequence analysis (Jakes *et al.*, 1991). These begin 14–16 residues downstream from the beginning of the first or second tandem repeat. The C-terminal extent of the unit, measured by mAb 423, is 93/95 residues, which is the same length as three repeats but the unit is 14–16 residues out of phase with respect to the maximum homology alignment proposed by Goedert *et al.* (1989b). This pattern is also out of phase with the linker-binder domain organization of the tandem repeat region proposed by Butner and

Kirschner (1991). Thus the folding of the repeat region within the core PHF does not appear to reflect the same organization as the full length tau molecule.

Production of the 12 kDa tau unit by proteases appears to depend on structural constraints within the PHF assembly itself. Once released from the PHF, it is susceptible to Pronase. Attempts to produce a similar fragment proteolytically from any of the isoforms of normal full length expressed or native tau result only in the complete degradation of the whole tau molecule (M.Novak, unpublished results). This suggests that production by proteases of the minimal resistant tau unit recognized by mAb 423 depends on an assembled configuration of tau found in either PHF or its precursor. This property provides the basis of an immunochemical assay which can be used to measure PHF accumulation in Alzheimer's disease brain tissue homogenate (Harrington *et al.*, 1991; Mukaetova-Ladinska *et al.*, 1992).

A question of fundamental importance for understanding the origin of the PHF is whether the C-terminal truncation of tau detected by mAb 423 reflects an endogenous event in PHF assembly. We have previously reported that mAb 423 labels intracellular neurofibrillary tangles, dystrophic neurites and granulovacuolar degeneration in Alzheimer's disease (Bondareff *et al.*, 1990, 1991; Mena *et al.*, 1991). We have also recently identified labelling of prominent pathological granular material within neurons lacking neurofibrillary tangles (M.Novak and C.M.Wischik, unpublished observation). The presence of mAb 423 immunoreactivity in all of the characteristic pathological inclusions of Alzheimer's disease suggests either that truncation of tau at Glu391 is produced by endogenous proteases as one of the events leading to PHF assembly, or that it reflects the action of proteases on a particular configuration of tau in a partially assembled precursor.

Materials and methods

Preparation of 12 kDa PHF core tau fragments

The isolation of the pronase treated PHF-core preparation (if II) and the 12 kDa PHF core tau (F5.5) preparation have been described previously (Wischik *et al.*, 1988a).

RNA extraction

Total RNA was isolated from the hippocampus and temporal lobe of a patient who had died with a histologically confirmed diagnosis of Alzheimer's disease (6 h *post mortem* delay) with acid guanidinium isothiocyanate as described by Chomczynski and Sacchi (1987). Poly(A)+ RNA was isolated by oligo(dT)−cellulose chromatography (Quick Prep mRNA Purification Kit, Pharmacia LKB Biotechnology) used later as AD mRNA in PCR.

Primers, PCR and DNA sequencing

14 cDNAs were prepared from AD mRNA using specific primers by PCR using reagents and conditions described in GeneAmp RNA PCR Kit (Perkin Elmer Cetus). A mock cDNA synthesis was performed by deleting AMV reverse transcriptase from the reaction mix and was used as a negative control template for subsequent PCR analysis. As sense primer for PHF core tau analogues the following sense primers were used: a, 48mer oligonucleotide 5′-GCCCGGGCCCCATATGCACCAGCCGGGAGGCGGGAAGGTGC-AAATAGT-3′; b, 47mer oligonucleotide 5′-GCCCGGGCCCCATATG-CTGAAGCACCAGCCGGGAGGCGGGAAGGTGC-3′; c, 49mer oligonucleotide 5′-GCCCGGGCCCCATATGCACGTCCCGGGAGGCGGCA-GTGTGCAAATAGTC-3′; d, 49mer oligonucleotide 5′-GCCCGGGCC-CCATATGATCAAACACGTCCCGGGAGGCGGCAGTGTGCAA-3′; e, 47mer oligonucleotide 5′-GCCCCATATGCACCAGCCGGGAGGCGG-GAAGGTGCAGATAATTAATA-3′. The following antisense primers were used for analogues ending at: KAKT, 47mer oligonucleotide 5′-AGAT-TACAGAATTCTCATGTCTTGGCTTTGGCGTTCTCGCGGAAGG-T-3′; GA, 46mer oligonucleotide 5′-AGATTACAGAATTCTCACGCC-CCGTGGTCTGTCTTGGCTTTGGCGT-3′; GAE, 47mer oligonucleotide 5′-AGATTACAGAATTCTCACTCCGCCCCGTGGTCTGTCTTGGCT-TTGGC-3′; GAEI, 47mer oligonucleotide 5′-AGATTACAGAATTCTC-AGATCTCCGCCCCGTGGTCTGTCTTGGCTTT-3′; KSPV, 49mer oligonucleotide 5′-AGATTACAGAATTCTCACACTGGCGACTTGT-ACACGATCTCCGCCCCGT-3′; The sense primer for an additional tau fragment was a 49mer oligonucleotide 5′-GCCCGGGCCCCATATGAG-CAAGGTGACCTCCAAGTGTGGCTCATTAGGC-3′ and the antisense primer was as for GAE (see above).

PCRs were carried out using a Techne PHC-3 thermal cycler (Duxford, UK) for 30 cycles (72°C, 1 min; 94°C, 1 min). The amplification product was cloned into pBluescript II KS+/− (Stratagene) and sequenced by the dideoxynucleotide procedure of Sanger *et al.* (1977).

Expression constructs

PCR products were gel purified, cleaved with *Nde*I and *Eco*RI and subcloned into *Nde*I- and *Eco*R-cut expression plasmid pRK 172 downstream of the T7 RNA polymerase promoter (McLeod *et al.*, 1987).

Bacterial expression and purification of candidate PHF core tau analogues

Recombinant plasmids were transformed into *Escherichia coli* Bl 21 (DE3) cells (Studier *et al.*, 1990). One litre cultures of *E.coli* were grown to an OD_{600} of 0.6−0.8 and with 0.4 mM IPTG for 3 h. Expression was checked by pelleting 1.5 ml of culture, immersing the pellet in liquid nitrogen for 5 min, resuspending it in 50 μl sample buffer (Laemmli, 1970) and running 5 μl on SDS−PAGE gel (10−20%). Following transfer the expressed PHF core tau analogues were identified using the generic anti-tau monoclonal antibody 7.51 (Novak *et al.*, 1991). The large-scale purification of tau fragments was carried out as described by Goedert and Jakes (1990).

Analysis of PHF core tau analogues by SDS−PAGE, immunoblotting and sequencing

Expressed, purified candidate tau analogues were analysed by SDS−PAGE (Laemmli, 1970) on 10−20% gradient minigels (Matsudaira and Burges, 1978). Transfer of bands for immunoblotting was carried out in 10 mM Caps buffer (pH 12.0) for 1 h at 145 mA. Following transfer, the PVDF membrane was treated with 1% (w/v) gelatin in phosphate-buffered saline (pH 7.4) and incubated for 4 h at room temperature with specific anti-PHF core monoclonal antibody mAb 423 (Novak *et al.*, 1991). Bound antibody was detected by the peroxidase technique using 4-chloro-1-naphthol (Sigma) as the chromogen. For sequence analysis bands were transferred to PVDF membrane (Matsudaira, 1987) stained with Coomassie blue, excised and sequenced using an Applied Biosystems gas-phase sequencer with on-line HPLC detection of phenylthiohydantoin derivatives of amino acids.

Miscellaneous

The hybridoma NOAL 6/66.423.2, which produces mAb 423, and hybridoma NOAL 7.51 secreting mAb 7.51 have been described previously (Novak *et al.*, 1989, 1991). Immunoelectron microscopy was carried out as described by Wischik *et al.* (1988a) using purified mAb 423. Immunohistochemistry was carried out as described in Bondareff *et al.* (1990).

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We thank R.Hills for tissue dissection and histology, Mrs P.Edwards for immunoelectron microscopy. Human brain tissue was obtained from the MRC Cambridge Brain Bank Laboratory. C.M.W. is a Lister Institute Research Fellow. We are indebted to ICI plc and ICI Americas, Inc., for financial support.

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EXHIBIT 3

DC11: a novel monoclonal antibody revealing Alzheimer's disease-specific tau epitope

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Using tau protein extracts from Alzheimer's disease (AD) brain tissue, we generated a monoclonal antibody (mAb DC11) which decorated neurofibrillary pathology in brain derived from AD patients on immunohistochemistry, and which lacked reactivity with healthy brain tissue. The same pattern of DC11 specificity was observed on Western blot. The main constituent of structures decorated by DC11 is microtubule-associated protein tau. In Western blot, DC11 recognized neither native healthy tau nor its full length recombinant counterpart. However, the mAb showed strong immunoreactivity with truncated tau (residues τ151–421), thus indicating the requirement for a conformational epitope. Importantly, the DC11 epitope was phosphorylation independent. The immunochemical parameters of mAb show that DC11 could represent a novel structural probe with the specificity for conformation of pathological tau present in AD brains. *NeuroReport* 14:000–000 © 2003 Lippincott Williams & Wilkins.

Key words: Alzheimer's disease; Conformation; Monoclonal antibody; Neurofibrillary pathology; Tau protein

INTRODUCTION

Alzheimer's disease (AD) is the most frequent cause of premature irreversible cognitive decline in adult life. There is a correlation between the degree of dementia and the content of pathological lesions: neurofibrillary tangles, neuritic plaques and neuropil threads. The common term for these pathological structures is neurofibrillary pathology. Molecular studies of lesions have demonstrated that their constituents are paired helical filaments (PHF) [1,2]. The major protein subunit of PHF is the microtubule-associated tau protein [3]. However, the conformation of tau involved in neurofibrillary pathology is distinct from that of normal healthy tau. Consequently, much effort was devoted to the generation of Alzheimer's tau specific monoclonal antibodies (mAbs). Currently, two groups of anti-tau mAbs are broadly exploited for analysis of AD-tau: phosphorylation-dependent antibodies and phosphorylation-independent antibodies. The first group of antibodies (e.g. AT8, AT100, PHF1, AP422) recognizes phosphorylated epitopes (phosphoepitopes) on tau protein [4–7], while the second group (e.g. mAb 423, ALZ-50 family) is specific for unphosphorylated tau epitopes [8,9]. In spite of great effort, at present there are no mAbs available for immunohistochemical diagnosis which are able to decorate exclusively neurofibrillary pathology in AD brains. All commercial phosphorylation-dependent anti-tau antibodies react with AD-associated phosphoepitopes, which could be demonstrated not only on diseased tau in AD brains but also on normal tau in cell cultures [10]. Phosphorylation-independent mAb ALZ50 exhibits in Western blot cross-reactivity with healthy human tau [11], and affinity parameters of mAb 423 are not optimal for histochemical application. Therefore the aim of our study was to develop monoclonal antibody with genuine specificity for neurofibrillary pathology in AD brains, which could be effectively used in immunohistochemistry and Western blot. Here we describe the preparation and characterization of a novel anti-tau mAb designated DC11.

MATERIALS AND METHODS

Immunogen: Preparation of immunogen from human AD brains was based on the method described by Köpke *et al.* [12], with the following modifications. Brain tissues from hippocampus of three AD pure (non-mixed) dementia patients (stage VI according to Braak's classification [13]) were homogenized (10% w/v homogenate) in 20 mM Tris pH 8, 0.32 mM sucrose, 10 mM β-mercaptoethanol, 5 mM EGTA, 10 mM EDTA, 5 mM $MgSO_4$, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 5 mM benzamidine, 5 µg/ml leupeptin, 1.5 µg/ml pepstatin, 2 µg/ml aprotinin in a Heidolph DIAX 900 homogenizer for 10 min at 4°C. The homogenates were pooled and centrifuged at

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27 000 × g for 30 min at 4°C to remove cellular debris. The pellet was resuspended in 100 mM Tris, pH 6.8, 20% glycerol, 4% sodium dodecylsulphate (SDS), 10 mM β-mercaptoethanol, centrifuged at 27 000 × g for 30 min at 4°C and the supernatant (AD brain extract) was dialysed against phosphate-buffered saline (PBS) overnight at 4°C. AD brain extracts were used for immunization of mice and further in Western blot analysis. The same method was used for preparation of brain extracts from control healthy age-matched human brains.

Immunization and fusion: Six-week-old female BALB/c mice (16 in total) were immunized with five i.p. injections of 50 µl AD brain extract emulsified in Freund's complete adjuvant (1:1) in the first dose and Freund's incomplete adjuvant (1:1) in the subsequent doses. Injections were done at 3–4 week intervals. The mice were boosted by an i.v. injection of the same amount of antigen without adjuvant. Harvested immune spleen cells were fused with mouse myeloma cell line NS0, following the fusion protocol described previously [14]. Growing hybridomas were screened by immunohistochemistry for production of monoclonal antibodies with specificity to decorate neurofibrillary pathology in AD brain tissue. Positive clones were recloned in soft agar. Hybridomas were grown in high glucose (4.5 g/l) Dulbecco's modified minimal essential medium (Sigma) supplemented with 10% horse serum and their culture supernatants used for experiments. Isotypes of mAbs were determined by an enzyme-linked immunosorbent assay (ELISA) using mouse Ig-isotyping kit (ISO-2, Sigma).

Recombinant human tau proteins: The cDNAs coding for six human tau isoforms were kindly provided by Dr M. Goedert (MRC-LMB Cambridge, UK). The cDNA for double truncated tau (residues τ151–421) was derived from mRNA isolated from the hippocampus of an AD patient by PCR using specific primers. The numbering of amino acids is that of the longest human tau isoform, τ40 [15]. All DNA constructs were cloned in *pET17b* vector (Novagen) through *Nde*I–*Eco*RI restriction sites. The integrity of each construct was verified by DNA sequence analysis (ABI Prism 377 DNA Sequencer, PerkinElmer). Tau cDNAs were expressed in *Escherichia coli* and recombinant tau proteins purified from bacterial lysates according to methods described elsewhere [16,17].

Immunohistochemistry: Brain tissues from AD patients and age-matched healthy persons were obtained from Slovak Brain Bank, Bratislava and Netherlands Brain Bank, Amsterdam (Table 1). Tissues from entorhinal cortex and hippocampus were fixed in 4% paraformaldehyde for 2 days, placed in 20% sucrose until they settled and then quickly frozen. The 50 µm thin sections were cut from frozen brains on Leica cryostat. Non-specific binding was blocked by preincubation of sections in a solution containing 5% normal horse serum and 0.3% Triton in PBS. Sections were incubated with mAb DC11 or with anti-tau phosphorylation dependent antibody AT8 (Innogenetics; 1:100 dilution in 0.3% Triton, PBS, 5% horse serum) overnight at 4°C. After washing, sections were incubated with horse anti-mouse Ig

Table 1. MISSING

The tissue samples were obtained from the Slovak Brain Bank and Netherlands Brain Bank. All cases were classified according to Braak's staging procedure [13]. Brain tissues used in this study had post mortem delay of 2.5–9.0 h. PMD, post-mortem delay; AD, Alzheimer's disease; pre-AD, preclinical AD without cognitive impairment; CERAD, Consortium to Establish a Registry for Alzheimer's Disease.

biotinylated antibody (Vector Labs) for 60 min, followed by avidin–biotin complex (Vector Elite, Burlingame, CA) and visualized with VIP solution (Vector VIP kit, Burlingame, CA). Sections were mounted on gelatin-coated slides, dried, treated with graded alcohols, xylene and coverslipped with Entellan (Merck, Darmstadt, Germany). For fluorescent labeling, sections were pretreated with 1% $NaHBO_4$ for 30 min to reduce brain tissue autofluorescence. Then stained with the monoclonal antibody DC11 and incubated with a horse anti-mouse biotinylated antibody (1:1000, Vector Lab., CA), followed by streptavidin-Alexa 488 (1:1000, Molecular Probes). Fluorescence was observed using the Olympus BX 51 fluorescent microscope. Controls without primary antibodies were included for each experiment.

Immunocytochemistry: For immunocytochemical analysis, cultures of hippocampal neurons were prepared from SHR rats (spontaneously hypertensive rat) at gestational day 18. Cells were plated on laminin-precoated glass slides (5 × 10^5 cells/slide) and cultivated for 5 days. Cultures were then fixed with 4% paraformaldehyde (Sigma) for 10 min at room temperature and cell membranes were permeabilized by incubation in 0.1% Triton X-100 in PBS for 4 min at room temperature. Cells were incubated with mAb DC11 or mAb AT8 for 1 h at RT. After washing, cells were incubated with biotinylated secondary anti-mouse Ig serum (Vector Labs) and then with streptavidin-ALEXA 488 (Molecular Probes). Preparations were examined with a confocal microscope Olympus IX 70. The same procedures were used for immunocytochemical study with BDNF (brain derived neurotrophic factor) differentiated human neuronal cell line SH-SY5Y [18].

Western blot analysis: Extracts from human control brains and AD brains were diluted in SDS-sample buffer, boiled and loaded onto 5–20% SDS polyacrylamide gel and electrophoresed in Tris–glycine–SDS running buffer for ~40 min at 25 mA. Transfer of proteins to a polyvinylidene fluoride (PVDF) membrane was performed for 1 h at 150 mA in 10 mM 3-(cyclohexylamino) propanesulfonic acid (CAPS), pH 12. After blocking with 3% non-fat dried milk in PBS for 1 h at room temperature, the membrane were incubated for 90 min with mAb DC11, Tau 1 (generous gift from Professor L. Binder) and pan-tau monoclonal antibody MN7.51 [19] (culture supernatants) respectively, followed by three washes with large volume of PBS for 5 min. Peroxidase conjugated goat anti-mouse Ig (DAKO) diluted 1:4000 with PBS were used as secondary antibody. Incubation (1 h at room temperature) was followed by washing (three times

with 0.2% Igepal in PBS). Positive reaction was detected by enhanced chemiluminescence (ECL) method (Amersham).

RESULTS AND DISCUSSION

The aim of our work was to develop a novel monoclonal antibody with potential application for neurofibrillary pathology analysis in AD brain tissues. Specifically, we looked for an antibody recognizing the AD-specific and not only AD-associated epitope. A monoclonal antibody with such immunochemical parameters has not been prepared yet. For generation of the required antibody we immunized mice with hippocampal extracts from AD brains. Following the 16 fusions, 5467 growing hybridomas were screened by immunohistochemical assay for production of mAbs with capacity to stain neurofibrillary pathology in AD brain tissues. In the second round of screening, 83 positive antibodies were tested for potential cross-reactivity with normal brain tissues from healthy persons. From those only one hybridoma clone secreted monoclonal antibody with AD-specific reactivity thus qualifying for further analyzis in Western blot. The clone was designated DC11 and produced antibody was of IgG1-isotype, which gave specific results in both assays. Recloning twice in soft agar stabilized antibody secretion of this hybridoma. Culture supernatant containing monoclonal antibodies was used for further analysis.

In immunochemistry, the antibody DC11 stained intensively and in highly specific manner neurofibrillary tangles, neuropil threads, ghost tangles and neuritic plaques which are present in AD brains (Fig. 1). No DC11 reactivity was observed with brain tissue form age-matched healthy persons (Fig. 2a,b). It is known that the major constituent of neurofibrillary pathology is tau protein [1], therefore we supposed that the DC11 epitope could result from AD-specific pathological conformation of tau. To prove this tempting possibility, we compared the immunoreactivity of mAb DC11 with the commercially available mAb AT8. The monoclonal antibody AT8 is directed against the phosphorylation-dependent epitope (phosphoepitope) on tau protein and has also ability to decorate neurofibrillary pathology [5]. In our study, both antibodies recognized neurofibrillary lesions in AD brain tissues albeit with different staining pattern (Fig. 2c,d). It was suggestive that the antigenic structure against which DC11 was raised could be formed on diseased tau protein. Further detailed analysis showed that, there is a significant difference in the specificity between the novel mAb DC11 and mAb AT8. The latter antibody, as well as other phosphoepitope-specific mAbs (AT100, PHF1, AP422) routinely used for staining of AD neurofibrillary lesions, binds the AD-associated phosphoepitope formed on pathologically hyperphosphorylated tau, but the same epitopes could be demonstrated also on normal phosphorylated tau [7,10,20]. This difference is evident from comparison of DC11 immunorectivity with that of AT8 on rat primary cultures (Fig. 2e,f). Whereas AT8 strongly reacted with the normal phosphorylated tau, DC11 showed no cross-reactivity with rat neurons. This type of cross-reactivity with normal rat tau we observed also with mAbs AT100 and PHF1 (not shown). The lack of DC11 reactivity with rat tissue provided a direct proof that DC11-

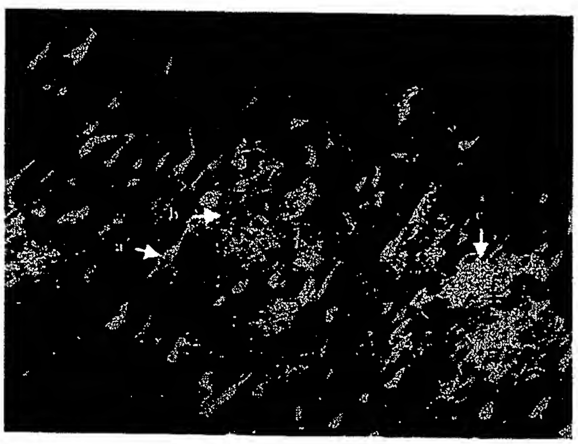

Fig. 1. Monoclonal antibody DC11 is able to recognize neurofibrillary tangles (arrow a), neuropil threads (arrow b) and neuritic plaques (arrow c) in AD brain tissues. Note the large number of neurofibrillary tangle-bearing pyramidal neurons stained in the hippocampal area CA 1. Bar = 50 μm.

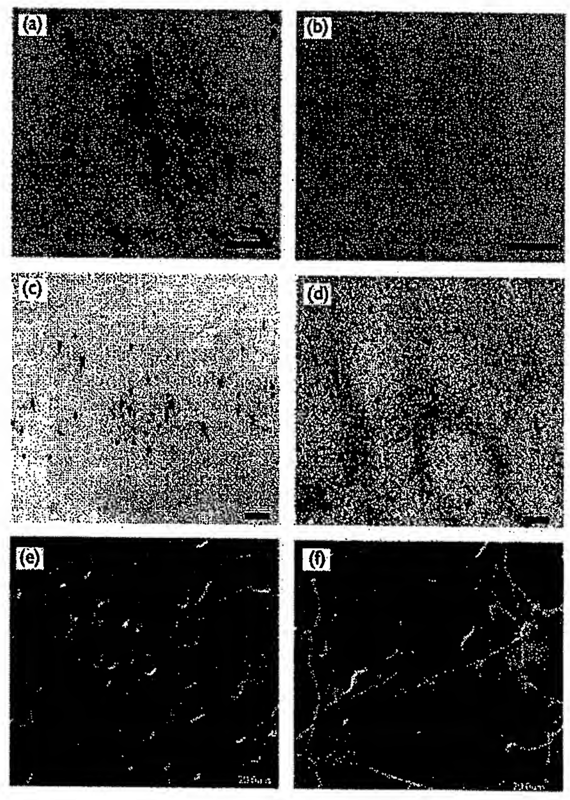

Fig. 2. Monoclonal antibody DC11 identified neurofibrillary pathology in the enthorinal cortex in AD brain (a) but not in healthy age-matched control brain (b). Moreover there was a significant difference in staining patterns between monoclonal antibody DC11 (c) and AT8 (d) on serial sections from AD entorhinal cortex. Normal tau in primary rat hippocampal cultures was not recognized with DC11 (e); however, AT8 immunoreactivity appeared mainly in axons and to the less extent in the perinuclear cytoplasm (f). Bar = 100 μm (a,b), 20 μm (c–f).

epitope is distinct from epitopes of other anti-tau mAbs compared in this study. In order to exclude possible species-specificity of DC11 we have tested its reactivity with human neuronal cell line SH-SY5Y, with the same negative result. Based on our findings, we suggest that the DC11-epitope is Alzheimer's tau specific and is not present on healthy tau protein. To confirm this suggestion, we have used for further DC11 epitope identification brain tau extracts from normal healthy age-matched controls; AD brain tissues and from six human recombinant tau isoforms. Indeed, Western blot analysis confirmed immunohistochemical findings and showed that mAb DC11 is able to discriminate between AD and healthy brain tissues (Fig. 3; Lane 1 and 2). The antibody showed high specificity for tau protein derived from AD brains and did not recognize the tau protein extracted from a normal human brain or recombinant human tau isoforms. Nevertheless, the unambiguous evidence that DC11 is able to react with tau protein, though structurally modified, was still missing. It has been suggested that to acquire AD-specific conformations, tau protein should undergo truncation [8]. Therefore we tested immunoreactivity of DC11 in Western blot with double truncated tau protein containing residues τ151–421 (Fig. 4). Surprisingly, while lacking reactivity with full length tau, the antibody showed strong binding with the truncated tau. Further, this observation shows that the epitope recognized by DC11 is conformational and its formation requires truncation of tau. Importantly, the reactivity with bacterially produced recombinant tau protein demonstrated that the epitope for DC11 was phosphorylation independent. This characteristics clearly distinguished DC11 from a group of phosphoepitope-specific anti-tau antibodies. In addition, it is interesting to note, that two phosphorylation-independent mAbs recognizing AD-tau (mAb 423, ALZ-50), have both conformational epitopes, as well. However, in contrast to DC11, mAb 423 recognizes only tau C-terminally truncated

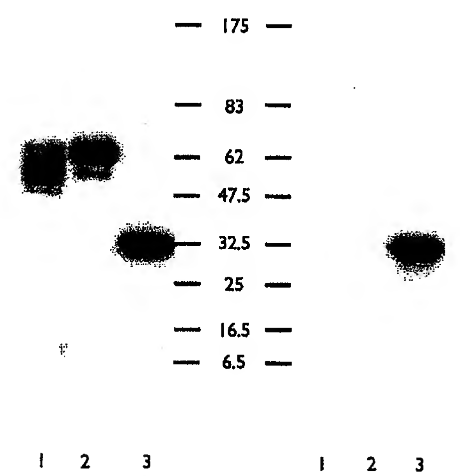

Fig. 4. Western blot analysis of full length recombinant tau40 (residues τ1–441) and tau fragment comprising residues τ151–421 demonstrated that only tau truncation could produce a conformation required by DC11. A control blot reacted with pan-tau mAb 7.51. Lanes: 1, six human recombinant tau isoforms; 2, full length recombinant tau 40 (residues τ1–441); 3, double truncated recombinant tau fragment composed of residues τ151–421.

at position Glu391 [8,19], whereas for binding of Alz50-family the N-terminal residues 2–9 of normal tau are necessary [9]. Thus it is evident that the novel antigenic structure defined on AD-tau by DC11 is distinct also from those identified by other phosphorylation-independent antibodies.

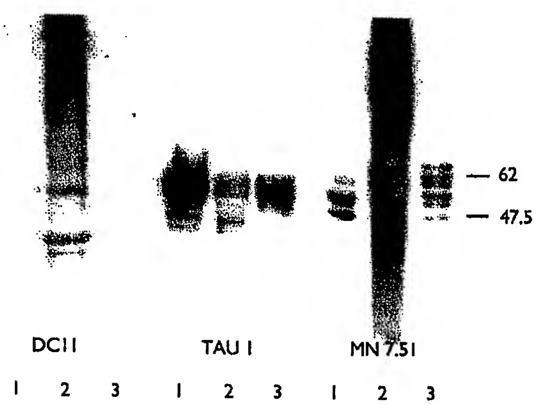

Fig. 3. In Western blot mAb DC11 recognized AD tau extracted from AD brains but did not react with tau extracted from healthy age-matched controls and with six human tau isoforms produced in bacteria. Two widely used anti-tau mAbs, Tau 1 and MN 7.51, stained healthy (lane1), diseased (lane 2) and recombinant (lane 3) tau proteins. Lanes: 1, tau extract from human healthy brain; 2, tau extract from AD brain; 3, six human recombinant tau isoforms.

CONCLUSIONS

We have produced the first-generation mAb, DC11 that can specifically discriminate between AD specific neurofibrillary changes and normal healthy brain tissues. Furthermore, biochemical and immunological analysis of tau protein extracts from normal healthy brains, AD diseased brains and recombinant human tau isoforms proved that mAb DC11 is recognizing conformationally modified Alzheimer's disease tau not present in normal healthy tau proteins. Identification of a novel antigenic structure on Alzheimer's tau will open new possibilities for detailed analysis of tau modifications specific to Alzheimer's disease. Furthemore, DC11 could be instrumental in dissecting the sequence of events leading to conformational transition of normal tau into AD-specific variant.

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EXHIBIT 4

Figure 1

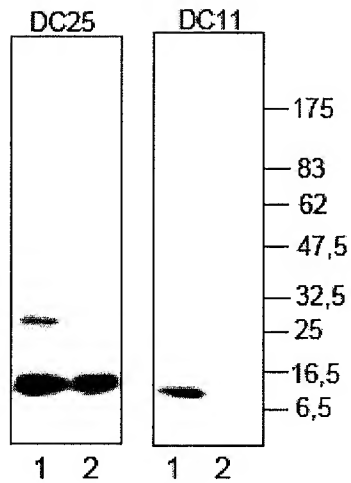

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Figure 2